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Review

The dynamics of the MBP–MalFGK$_2$ interaction: A prototype for binding protein dependent ABC-transporter systems

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Abstract

This review is focused on the interaction between maltose binding protein (MBP) and the maltose transporter complex, MalFGK$_2$, which is a member of the ATP Binding Cassette (ABC) superfamily. The interaction between MBP and MalFGK$_2$ has a critical role in maltose transport, but a coherent description of the interaction is complicated because both MBP and MalFGK$_2$ can adopt multiple conformations. Drawing on genetic, structural, and biochemical data, the different conformations of MBP and MalFGK$_2$ are described and incorporated into a model for their interaction. The most important feature of this model is that ligand-bound MBP initiates the process of ATP-dependent maltose transport by stabilizing a high-energy conformation of MalFGK$_2$. In this model of the MBP–MalFGK$_2$ interaction, stabilization of a high-energy conformation of MalFGK$_2$ allows ATP to drive conformational changes in the system – in particular the opening of bound MBP – that leads to formation of a transition state for ATP hydrolysis. Such a role for ligand-bound MBP explains how MBP-independent MalFGK$_2$ mutants work, and represents a general mechanism for binding-protein dependent ABC import systems. In ABC export systems, which do not use a binding protein, the substrate itself is expected to play a role similar to ligand-bound MBP in the maltose transport system. The mechanistic model for the maltose transporter suggests that ABC-type import systems evolved to make use of a peripheral binding protein so that the transport process is essentially irreversible.

Keywords: ABC transporter; Maltose binding protein; Chemical–mechanical coupling; Reversibility; Protein–protein interaction; ATP hydrolysis; Transmembrane transport; Binding energy; Transition state stabilization

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Periplasmic binding proteins (PBPs), and related membrane-bound lipoproteins of Gram-positive organisms, are major components of the bacterial cell envelope. In addition to their functions in the bacteria, it was recognized many years ago that the glutamate binding subunit of G-protein coupled and ionotropic glutamate receptors bore sequence similarity to the

Abbreviations: ABC, ATP binding cassette; ABP, Arabinose binding protein; MalFGK$_2$, Maltose transporter integral membrane complex; MBP, Maltose binding protein; MBPi, MBP-independent; MM, Minimal maltose; PBP, Periplasmic binding protein; PDB, Protein data bank; SCOP, Structural classification of proteins

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glutamine binding protein of *Escherichia coli* [1,2]. As genome sequences have continued to emerge, it is clear that the basic PBP structure and binding mechanism has been used in a variety of contexts in eukaryotic organisms [3,4].

The PBPs have been grouped into 8 families based on sequence analysis [5]; however, a wealth of structural data indicate that most of these proteins can be described by one of two topologies, catalogued in the SCOP database as periplasmic binding protein-like superfamilies I and II [6]. Proteins in both superfamilies typically contain two α/β domains connected by a linker consisting of two or three *β*-strands; in all cases, ligand is bound in the cleft between the two domains. A comprehensive review of the structures and ligand binding mechanisms for representative members of these two superfamilies is available [7]. A third group of binding proteins has also been identified from the structures of the Zn^{2+}-binding protein TroA from *Treponema pallidum* [8], as well as the iron-siderophore binding protein, FhuD [9] and vitamin B12 binding protein, BtuF [10], both from *E. coli*. As with other PBPs, these proteins serve as the ligand-binding subunits for ABC transport systems, but in contrast to other PBPs they contain a long α-helical insertion in the interdomain linker and have a more shallow ligand binding cleft.

The primary function of a PBP is in nutrient uptake, and therefore the most important interaction of a PBP is with its cognate ABC transporter. The interaction between a PBP and its ABC transporter leads to hydrolysis of ATP and transport of the ligand – a carbohydrate, amino acid, peptide, or metal ion – into the cytosol. The PBP-dependent ABC import systems are fascinating because it is the binding protein, and not the substrate itself, that regulates the ATPase activity of the system. In other words, ATP hydrolysis does not appear to be directly coupled to solute transport, but rather to the interaction between the PBP and the ABC transporter. It is well known that ligand binding induces a conformational change in the PBP, but the mechanism by which the ligand-bound conformation of the PBP stimulates the ATPase activity of the ABC transporter remains a major question in the field. Resolution of this issue will help us to understand how ATP hydrolysis is regulated in ABC transporters in general, and may aid in the design of antibiotics that are actively acquired by bacteria. This review focuses on the prototypical member of the periplasmic binding protein superfamily, maltose binding protein (MBP) from *E. coli*, and its associated ABC transporter complex, $MalFGK_2$.

MBP holds its position as the most well studied PBP for several reasons. The first is that growth on minimal maltose (MM) media provides a simple and robust marker for a functional maltose transport system, greatly facilitating genetics-based investigations. Second, the requirement for secretion of MBP to the periplasm enabled studies of the general secretory system, and in this respect MBP continues to be used as a model secretion substrate and its stability and folding properties have often been important considerations in this research. While the main function of MBP is to act as the primary maltodextrin receptor for $MalFGK_2$, it also functions as the chemotactic receptor for maltose by interacting with the TAR chemoreceptor [11,12]. MBP interacts with the LamB porin, playing an essential role in the facilitated diffusion of longer maltodextrins across the outer membrane [13–15]. Its involvement in a variety of different systems resulted in widespread use of MBP as a research reagent, and its favourable physical properties contributed to the adoption of MBP as an affinity tag for protein expression and purification [16,17], as well as a platform for protein design and engineering [18].

1. MBP: the molecule

Crystal structures of liganded and unliganded MBP were solved by Quiocho and co-workers [19,20]. The interactions between MBP and maltose-based ligands have been described in detail by Quiocho's group through a number of high-resolution crystal structures [21–24]. For the purposes of this review, which is focused on the interaction between MBP and $MalFGK_2$, the most important observations were that the ligand bound in a cleft between two domains connected by a hinge, and that ligand binding brought about a large conformational change in the protein, both in the crystal [19] and in solution [25,26]. This ligand-induced conformational change from "open" to "closed" (Fig. 1) appears to be a general feature of the PBPs, and was dubbed the "Venus Fly-Trap Mechanism" [27] because of its resemblance to the traps on the carnivorous herb that close only when stimulated by prey.

Crystallographic models provide a structural "snapshot" in the sense that the protein must adopt a fixed conformation to form well-ordered crystals. In this regard, the structure of unliganded MBP in solution may be slightly different from its crystal structure. NMR studies of unliganded MBP showed it was slightly more closed than the crystal structure, whereas the conformation of maltotriose-bound MBP was essentially identical in solution and in the crystal [23,28]. A similar result was obtained from small-angle X-ray scattering experiments, in which there was a perfect match between the maltose-bound crystal structure and the structure in solution, but unliganded MBP in solution appeared to adopt a slightly more closed conformation than the crystallographic model [29]. Molecular dynamics simulations also found that the average structure for the unliganded protein was slightly more closed than the crystal structure [30].

In fact, the individual domains of unliganded MBP appear to be dynamic. Evidence for independent domain motions comes from tryptophan fluorescence anisotropy measurements, in which the loss of fluorescence anisotropy is measured and then modeled as the sum of several exponential decays, or "relaxation processes". These measurements indicated a relatively slow relaxation process in the unliganded protein that was absent in the maltose bound protein and also in the β-cyclodextrin bound protein [31]. The β-cyclodextrin bound protein was crystallized in an open conformation [32], and is only partly closed in solution [28]; therefore, the absence of the slow relaxation process in liganded MBP is not a function of cleft closure, but rather the interaction of the domains with the ligand. On this basis, the slow relaxation process observed in unliganded MBP was attributed to rigid body motions of the individual domains around the flexible hinge, although the full extent of these motions could not be

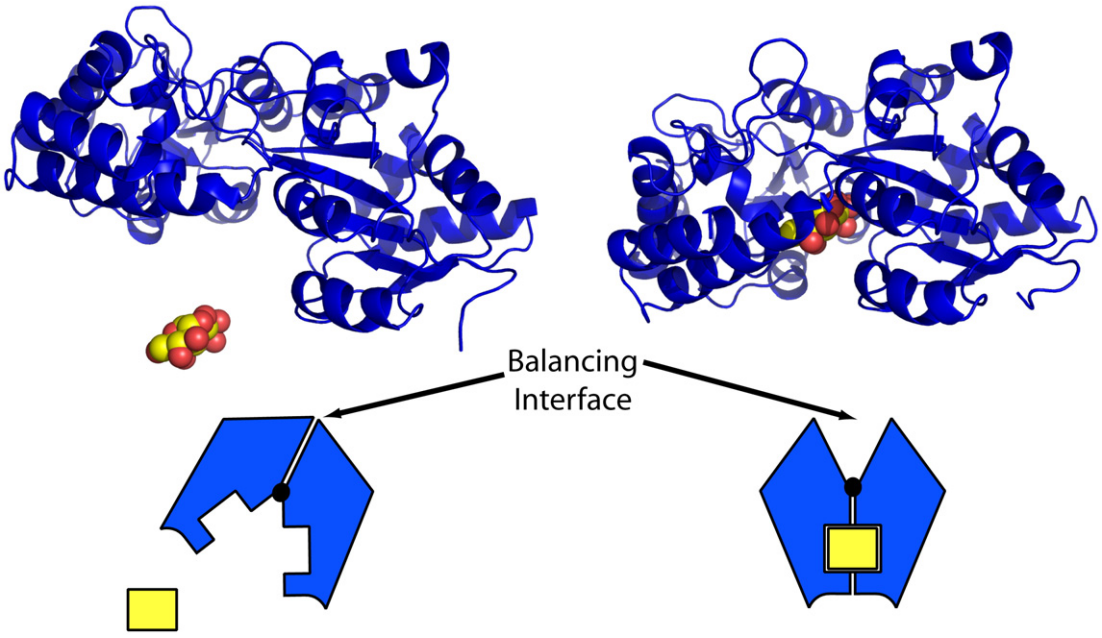

Fig. 1. Maltose binding protein. Maltose binding protein (MBP) is shown in the open (left; PDB ID 1OMP [19]) and closed (right; PDB ID 1ANF [20]) conformations, along with schematics that illustrate essential structural features, namely the ligand binding site, hinge, and balancing interface. The two domains of MBP undergo a rigid body rotation to enclose maltose (yellow square) in the binding cleft. As the binding cleft is closed, contacts in the balancing interface opposite the hinge (black circle) are broken.

determined from these measurements. These experimental results are consistent with molecular dynamics simulations in which the unliganded protein sampled a wider variety of conformations than the maltose-bound protein [30]. Thus, ligand binding not only affects the relative orientation of the two domains but also attenuates their movement relative to each other.

Given the dynamic nature of unliganded MBP, it is conceivable that a fully closed conformation might exist in solution, and this could help to explain the stimulatory effect of maltose-free MBP on the $MalFGK_2$ ATPase [33]. The open conformation of the protein is stabilized by interdomain interactions on the side of the hinge opposite the ligand binding cleft, a region we termed the "balancing interface" [29]. Disruption of interdomain interactions in the balancing interface was shown to increase the affinity of MBP for its ligands, presumably by destabilizing the open conformation [29,34]; however, the removal of balancing interface interactions had no effect on the solution conformation of the unliganded protein, arguing that there is very little, if any, closed MBP in the absence of ligand [29]. In the absence of a maltodextrin ligand, the closed conformation appears to be a high energy and largely unpopulated state. For example, a Zn^{2+}-binding site was carefully engineered into the substrate-binding cleft of MBP to stabilize the closed conformation [35] but Zn^{2+} binding had no detectable effect on the conformation either in solution or when crystallized [36], consistent with the closed conformation being inaccessible in the absence of maltose. A detailed analysis of the energetics of domain closure was carried out by introducing a series of bulky substitutions in the balancing interface. These substitutions forced the unliganded protein to adopt more closed conformations in solution, and at the same time caused a proportional decrease in the stability of the protein [37]. That is, in going from the open to closed conformation, the balancing interface is pulled apart and hydrophobic surface is exposed, which, in the absence of stabilizing interactions with maltose, decreases the overall stability of the protein. In fact, each degree of domain closure costs approximately 212 cal/mol in protein stability, suggesting that a fully closed, unliganded MBP would not be stably folded at 37 °C [37]. In summary, in solution and in the absence of ligand, the domains of MBP are dynamic, fluctuating around an average orientation that is only slightly more closed than the open conformation observed in crystals of unliganded MBP. When maltose binds to MBP, it interacts first with aromatic groups on the C-terminal domain to form a complex with the open form [22], which then converts to the stable closed conformation [38]. In terms of a general model for binding protein function, therefore, MBP follows the "Venus Flytrap Model" in which the protein adopts a stable closed conformation only in the presence of ligand [27,39].

2. Two functions for the MBP–$MalFGK_2$ interaction

The most important interaction of MBP is with the integral membrane subunits, MalF and MalG, of its cognate ABC transporter, $MalFGK_2$. Both MBP and $MalFGK_2$ can adopt multiple conformations and therefore they can interact in a number of different ways. In fact, for transport of maltose to occur, the MBP–$MalFGK_2$ interaction must be dynamic and lead to coordinated conformational changes in the complex. On this basis, the complex that is initially formed between maltose-bound MBP and $MalFGK_2$ must be relatively unstable such that it produces conformational changes in both molecules, thereby facilitating ATP hydrolysis and release of maltose from its high-affinity binding site in MBP. This situation resembles, in principle, the theory of transition state stabilization for enzyme catalysis [40–43]. The critical point is that in order for catalysis to proceed after the formation of the initial enzyme–substrate

complex, the enzyme must bind the transition state more tightly than the ground state of the substrate. If this were not the case, the enzyme–substrate complex would continue to exist as a low energy, stable, and inactive complex. With this in mind, the idea that ligand-bound MBP must interact with a high-energy conformation of $MalFGK_2$ needs to be considered and incorporated into models of their interaction.

The nature of the conformational changes in $MalFGK_2$ was first addressed by Howard Shuman when he isolated an *E. coli* mutant strain that could grow on minimal maltose (MM) media in the absence of MBP. There are several ways for *E. coli* to acquire the ability to grow on MM, but in one particular strain the mutation was closely linked to *malF* and therefore it represented the first "MBP-independent" (MBPi) mutant of $MalFGK_2$ [44]. As in the wild-type system, maltose transport in this strain was sensitive to arsenate, indicating that ATP hydrolysis was required for transport. Assuming a single substrate binding site in $MalFGK_2$, a model for MBP-independent maltose transport was described where ATP binding and hydrolysis drove conformational changes in $MalFGK_2$, resulting in alternating exposure of a single maltose binding site to the periplasm and to the cytoplasm (Fig. 2). The structural features of this model have been borne out by crystal structures of intact ABC transporters that indicate a single substrate binding site formed between two bundles of transmembrane helices, each of which is attached to an ATP binding cassette and presumably subject to ATP-driven conformational changes within and between these cassettes [45–48]. Although a crystal structure for intact $MalFGK_2$ is not available, structures of the isolated MalK dimer show that the ATP binding cassettes are loosely associated in the absence of nucleotide, and that ATP binding brings about a tighter association, closing the dimer interface [49]. ATP hydrolysis then allows the subunits to return to their initial loosely associated conformation [50]. Based on these observations, in addition to isolation and characterization of a MBP–$MalFGK_2$(ADP–vanadate) transition state complex [51,52], the conformation of $MalFGK_2$ with the substrate binding site facing the periplasm likely corresponds to the tightly-associated conformation of the ATP binding cassettes.

The observation that mutants of the $MalFGK_2$ importer can function without MBP, combined with the fact that ABC-type export systems work without a binding protein, raises the question of the functional role of MBP in the maltose transport system. In more general terms, why is it that ABC-type import systems have evolved to make use of peripheral binding proteins? The wild-type maltose transport system displays a K_M (defined as the maltose concentration that yields a half-maximal rate of transport) of 1 μM which is roughly the same as the K_D of MBP for maltose [53,54]. On the other hand, MBPi–$MalFGK_2$ mutant systems exhibit a low affinity for maltose, with a K_M of approximately 2 mM [44], over 3 orders of magnitude higher than the wild-type system. Thus, one function of MBP is to provide a high-affinity binding site for maltose. In terms of natural selection, it is conceivable that a maltose transport system could have evolved without a peripheral high-affinity binding protein, but instead with the high-affinity binding site in the integral membrane subunits. One obvious disadvantage to such a system is that there can be only a limited number transport complexes in the bacterial cell membrane. In contrast, MBP is expressed at very high levels in the *E. coli* periplasm – its concentration is estimated to be 1 mM [55] – and therefore by separating the maltose binding site from the integral membrane transporter, the system is able to capture many more maltodextrin molecules, which can be retained in the periplasm and fed to the smaller number of transporters.

Another function for MBP and its interaction with $MalFGK_2$ was discovered from *in vitro* ATPase measurements of $MalFGK_2$, in which it was shown that maltose-bound MBP had a strong stimulatory effect on the ATPase activity of $MalFGK_2$ [33]. In fact, even *unliganded* MBP was able to stimulate the $MalFGK_2$ ATPase, although at a much lower level than liganded MBP. The main conclusion from these experiments was that ligand-bound MBP functions as a signaling molecule to activate the $MalFGK_2$

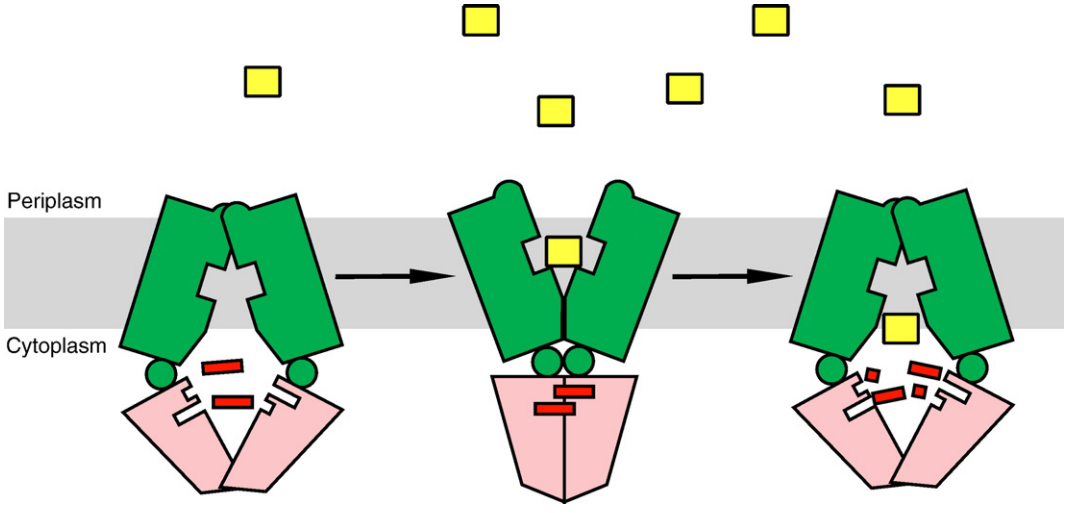

Fig. 2. MBP-independent transport by $MalFGK_2$. The mutations in MBP-independent $MalFGK_2$ complexes allow a high rate of ATP hydrolysis in the absence of MBP and/or maltose. A model for this process, based on genetic and structural data as outlined in the text, is that ATP binding and hydrolysis by the MalK subunits (pink) drive conformational changes in MalF and MalG (green), exposing the maltose binding site alternately to the periplasm and cytoplasm. If maltose (yellow squares) is present, it can be transported by the complex. ATP, ADP, and Pi are indicated by the long, medium, and short red bars, respectively.

ATPase. Similar *in vitro* ATPase measurements were carried out for MBPi–MalFGK$_2$ mutants, which exhibited a high rate of ATP hydrolysis irrespective of the presence of either MBP or maltose [33]. Thus, ligand-bound MBP activates ATP hydrolysis by wild-type MalFGK$_2$, and this second function of MBP can be replaced or mimicked by mutations in MalFGK$_2$.

This transmembrane signaling function of MBP is particularly interesting. In most active transport systems the energy that the system consumes is somehow coupled to the movement of the solute across the membrane. In other words, the solute itself binds to the transporter and triggers conformational changes in the system that lead to translocation and concomitant energy consumption. The maltose transporter appears to be different in that energy from ATP hydrolysis is not directly coupled to solute transport, but instead to the interaction between MBP and MalFGK$_2$ [33,52,56]. Maltose is effectively buried when bound to MBP and therefore the cycle of conformational changes in the system is initiated before maltose contacts MalFGK$_2$. The indirect role of maltose transport in energetic coupling is reinforced by the fact that even unliganded MBP can stimulate the MalFGK$_2$ ATPase to some degree [33], and therefore transport of maltose is apparently not required for ATP hydrolysis. This is in contrast to ABC export systems, in which ATP hydrolysis is stimulated by the ligand itself [57].

3. The MalFGK$_2$ interaction surface on MBP

The MBPi–MalFGK$_2$ mutant identified by Howard Shuman [44] served as the starting point for additional experiments that yielded important insights into MalFGK$_2$ dynamics and the nature of the MBP–MalFGK$_2$ interaction. The experiments were predicated on the intriguing observation that wild-type MBP, when added back to the strain carrying an MBPi–MalFGK$_2$ system, actually *inhibited* growth on MM [58]. This surprising result facilitated selection of additional MBP-independent mutants that map specifically to *malF* and *malG* [58]. The MBPi–MalFGK$_2$ mutants varied significantly in their doubling times when grown on MM, suggesting that they differed in their ability to transport maltose. The mutants all had transport K_M values of approximately 2 mM, but exhibited differences in their maximal rate of maltose transport. Furthermore, the ATPase activity of the different MBPi–MalFGK$_2$ mutants was proportional to their growth rate on MM [33]. To put these observations in the context of the single alternating site model for MBPi–MalFGK$_2$ systems (Fig. 2), the rate of maltose transport should be proportional to the rate of cycling between the open-to-periplasm and open-to-cytoplasm conformations, and therefore MBPi–MalFGK$_2$ mutants with the highest maltose transport, growth rates, and ATPase activity are expected to have the highest rates of conformational cycling.

The ability of wild-type MBP to inhibit growth of MBPi–MalFGK$_2$ strains, presumably by interacting non-productively with the MBPi–MalFGK$_2$ mutant complex, was used by Shuman and co-workers to characterize the MalFGK$_2$ interaction surface on MBP. The rationale was that the growth-inhibitory effect of MBP on MBPi–MalFGK$_2$ mutant strains could be suppressed by mutations in residues that are involved in the MBP–MalFGK$_2$ interaction [59]. In these experiments, strains carrying various MBPi–MalFGK$_2$ mutant systems were reconstituted with an MBP-expressing vector that had been subjected to random mutagenesis; strains were then selected based on their ability to grow on MM. One important observation was that many of the MBP suppressor mutants had simply lost the ability to bind maltose, indicating that the closed conformation of MBP was essential for the non-productive interaction with the MBPi–MalFGK$_2$ complex. A smaller group of MBP suppressor mutants – the so-called Group 1 mutants – were able to bind maltose with high affinity. Three of the suppressor mutations in Group 1 – G13D, D14Y, and Y210S – were located on the surface of MBP, near the edge of the maltose binding cleft. In the closed, ligand-bound conformation of MBP the mutations delineate a continuous surface or patch, but in the open conformation, this surface is broken (Fig. 3). The proximity of G13, D14, and Y210 in the closed conformation, combined with the observation that when mutated these three residues suppress the growth inhibitory effect of MBP on MBPi–MalFGK$_2$ mutants, was good evidence that they form the MBPi–MalFGK$_2$ interaction surface.

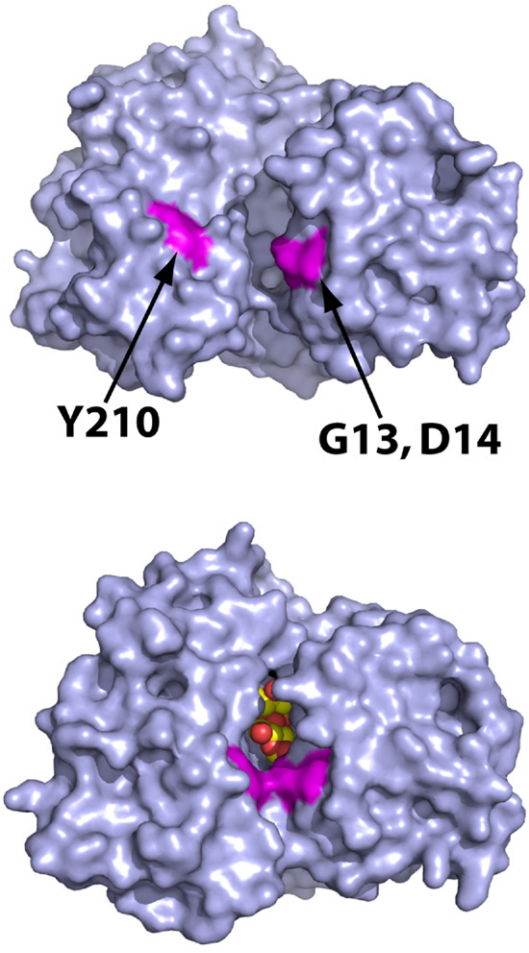

Fig. 3. The MalFGK$_2$ Interaction Surface of MBP. Surface representations of the open (top; 1OMP [19]) and closed (bottom; 1ANF [20]) conformations of MBP are shown, looking into the maltose binding cleft. Residues G13 and D14 on the N-terminal domain, and Y210 on the C-terminal domain, are involved in the interaction with MalFGK$_2$ [59,60].

Additional support for the idea that the region around Y210 is involved in the interaction with $MalFGK_2$ came from a separate set of experiments with wild-type $MalFGK_2$ [60]. Here, mutants of MBP were selected based on their ability to act as dominant negative inhibitors of the wild-type system, and in these experiments a Y210C mutant of MBP was repeatedly isolated. Upon further investigation, it was found that the Group 1 suppressor mutants of MBP acted as dominant negative inhibitors of wild-type $MalFGK_2$, while the Y210C dominant negative MBP acted as a suppressor for MBPi–$MalFGK_2$. In summary, residues G13, D14, and Y210 form a contiguous surface patch only in the closed conformation of MBP, and when mutated can result in an MBP molecule that inhibits transport by wild-type $MalFGK_2$ and can relieve inhibition of MBPi–$MalFGK_2$. Therefore these residues are involved in an interaction with $MalFGK_2$ that is critical for the regulation of ATP binding and hydrolysis.

4. Transition state stabilization by MBP

The interaction between MBP and $MalFGK_2$ plays a critical role in the transport process, and knowledge of the conformational dynamics of MBPi–$MalFGK_2$ mutants can be used as a starting point to incorporate MBP into a mechanistic model for maltose transport by the wild-type system. In the simplest case, the closed form of MBP would interact with the open-to-cytoplasm conformation of $MalFGK_2$, and the open form of MBP would interact with the open-to-periplasm conformation of $MalFGK_2$. Although straightforward, this model does not provide an attractive mechanism for progression through a series of conformational changes. In particular, ligand-bound MBP is in a stable, low-energy conformation, and therefore a high-affinity interaction with the "resting" or low energy conformation of $MalFGK_2$ (i.e. the open-to-cytoplasm form) would result in the formation of a stable MBP–maltose–$MalFGK_2$ complex. Since binding of maltose–MBP to $MalFGK_2$ triggers ATP binding and hydrolysis along with opening of MBP and release of maltose, the MBP–$MalFGK_2$ interaction must yield a relatively unstable complex that will progress to new conformations in both MBP and $MalFGK_2$.

For wild-type $MalFGK_2$, which has a very low rate of ATP hydrolysis in the absence of MBP, there must be a conformation that is intermediate between the open-to-cytoplasm and open-to-periplasm forms and represents a significant energetic barrier so that uncoupled ATP hydrolysis is prevented. Binding of maltose–MBP to such a high-energy conformation would act to stabilize it and lower the energetic barrier, allowing progression to the open-to-periplasm form of $MalFGK_2$. This idea is illustrated in the transport schematic in Fig. 4.

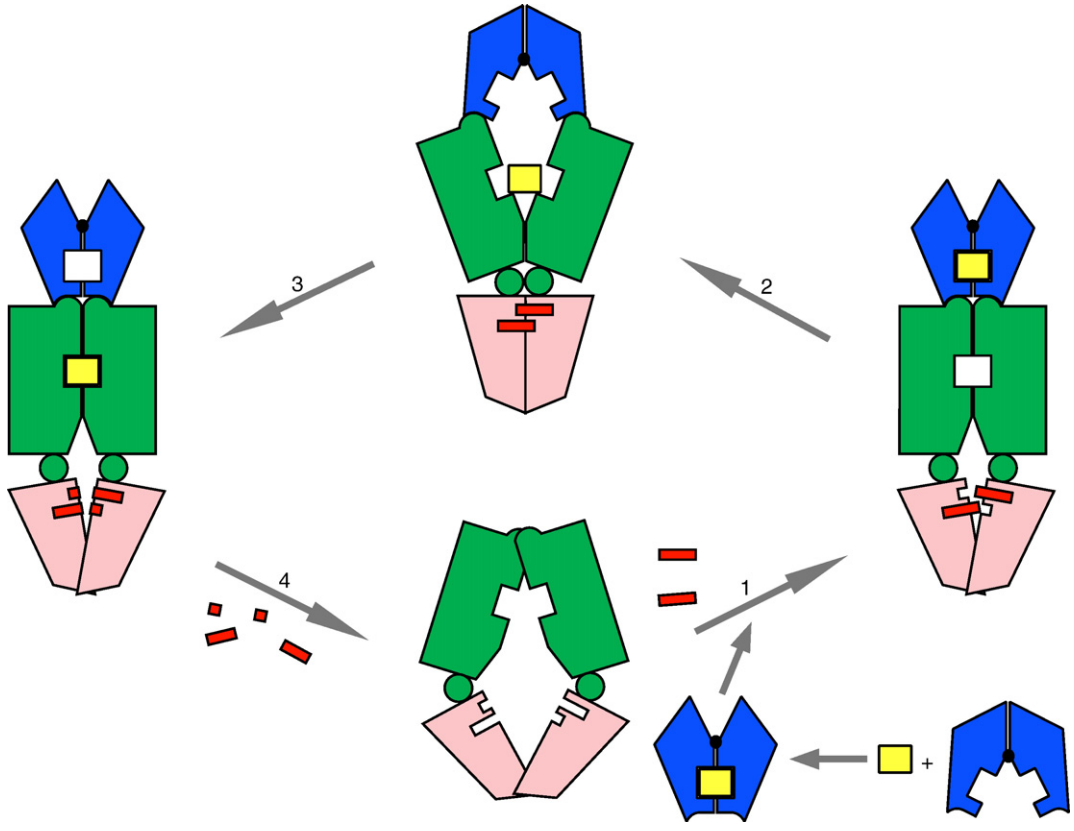

Fig. 4. Maltose transport mechanism: stabilization of a high-energy intermediate by MBP. MBP is indicated in blue, MalFG in green, and $MalK_2$ in pink; ATP, ADP, and inorganic phosphate are shown as long, medium, and short red bars, while maltose is indicated by the yellow rectangle. In the transport cycle, $MalFGK_2$ exits in a resting conformation with its substrate-binding site open to the cytoplasm and the nucleotide binding sites open and available for nucleotide binding and/or release (bottom). When $MalFGK_2$ has the substrate binding site open to the periplasm (top), the MalK subunits form a tight dimer with the ATP molecules occluded, non-exchangeable, and poised for hydrolysis — this conformation represents the transition state for ATP hydrolysis [51,52]. Conformations of $MalFGK_2$ that are intermediate between the open-to-cytoplasm and open-to-periplasm forms are proposed to be high-energy states stabilized by interaction with MBP. Mutations in $MalFGK_2$ that produce an MBP-independent phenotype reduce the energy of these conformations, allowing a high rate of ATPase activity in the absence of MBP.

In the reaction cycle in Fig. 4, the resting state of $MalFGK_2$ has the substrate binding site facing the cytoplasm, and the nucleotide binding sites are open and accessible; this conformation resembles what was observed for $ModB_2C_2$ in complex with its binding protein, ModA [45], and for the isolated $MalK_2$ dimer in the absence of nucleotide [49]. Binding of liganded MBP to $MalFGK_2$, in Step 1, will stabilize the intermediate, bringing the MalK subunits closer together such that the binding energy of ATP can promote tight dimerization. The ATP-driven close association of the two MalK subunits has been demonstrated in recent cross-linking studies of intact $MalFGK_2$ [61]. In Step 2, the ATP-driven dimerization of the MalK subunits opens the substrate binding site in $MalFGK_2$ to the periplasmic side and also opens MBP, allowing release of maltose [51,52]. Once ATP is hydrolyzed, the tight $MalK_2$ dimer is no longer stable and $MalFGK_2$ returns to its intermediate conformation (Step 3); the loosening of the $MalK_2$ dimer in the intact complex upon ATP hydrolysis is consistent with what has been observed for isolated $MalK_2$ as well as intact $MalFGK_2$ [50,61]. As $MalFGK_2$ returns to its resting conformation, bound MBP would be forced to close without its ligand, at some point dissociating from $MalFGK_2$.

In this reaction cycle, uncoupled ATPase activity is prevented because $MalFGK_2$ must pass through a high-energy intermediate; maltose-bound MBP promotes the ATP hydrolytic cycle by stabilizing this intermediate. On this basis, the constitutive ATPase activity of MBPi–$MalFGK_2$ systems indicate that the high-energy state is no longer present. In other words, the mutations in MBPi$MalFGK_2$ molecules have lowered the energy of the intermediate, increasing the likelihood of transitions between the open-to-cytoplasm and open-to-periplasm conformations. The mutations that allow MBP-independent transport are all found in the integral membrane subunits – either MalF or MalG – and never in the ATP binding cassette subunit, MalK [44,58,62]. In fact, it was shown that MBP-independent transport requires two mutations that act synergistically: one mutation is found in a region close to the predicted periplasmic surface of MalFG, and the second mutation is further away, often close to the centre of a transmembrane helix [62]. On the basis of the MBPi–$MalFGK_2$ mutants, therefore, the barrier to ATP hydrolysis is present in the integral membrane subunits of the transporter, and not the ABC subunits.

How does wild-type MBP inhibit MBPi–$MalFGK_2$ mutant systems? To answer this question there are three important considerations. The first is that the MBPi–$MalFGK_2$ mutants with the fastest growth rates were also the most sensitive to inhibition by MBP. Assuming that the alternating site model for the MBPi–$MalFGK_2$ mutants is correct (Fig. 2), this means that MBPi–$MalFGK_2$ mutants with a relatively high rate of cycling are the most susceptible to inhibition by MBP. In other words, ligand-bound MBP is interacting non-productively with a conformation of $MalFGK_2$ that only appears when it is cycling between the open-to-cytoplasm and open-to-periplasm conformations. The second consideration is that inhibition of MBPi–$MalFGK_2$ systems requires the closed, liganded form of MBP [59]. The third and final consideration is that the inhibition of maltose uptake occurs only at high maltose concentrations, such as the millimolar concentrations present in MM media. At micromolar maltose concentrations, MBP actually restores high-affinity maltose transport by MBPi–$MalFGK_2$ systems [63]. Therefore, it appears that saturation of the MBPi–$MalFGK_2$ system with maltose is necessary for inhibition by wild-type MBP. On the basis of these three considerations, the site of inhibition is the intermediate between steps 3 and 4 (Fig. 4): as the MBPi–$MalFGK_2$ system becomes saturated with maltose, this intermediate would contain two maltose molecules – one in MBP, and one in $MalFGK_2$ – and by stabilizing the closed form of MBP, the second bound maltose could lower the energy of the entire complex and slow the transition to the open-to-cytoplasm conformation.

5. The interaction between unliganded MBP and $MalFGK_2$

There is evidence that unliganded MBP interacts with $MalFGK_2$ and has an important role in promoting ATP hydrolysis. First, unliganded MBP stimulates the ATPase activity of wild-type $MalFGK_2$ [33]. Second, analysis of transport kinetics shows that the only models consistent with experimental data are those incorporating an interaction between unliganded MBP and $MalFGK_2$ [64,65]. This theoretical treatment was supported by experimental results in which increases in the concentration of MBP actually inhibited transport when the maltose concentration was held constant at a sub-stoichiometric level [66]; in other words, unliganded MBP appeared to be competing with liganded MBP for interaction with $MalFGK_2$. Third, a high-affinity interaction between unliganded MBP and $MalFGK_2$ at the ATP hydrolysis stage was demonstrated by isolation of a vanadate-trapped MBP–$MalFGK_2$ complex with MBP in its open conformation [51,52]. Finally, the idea that the interaction of open, unliganded MBP with $MalFGK_2$ is important for ATP hydrolysis is consistent with the engineering of a mutant $MalFGK_2$ system that was able to transport lactose, but only in the presence of MBP [67]. Since MBP cannot bind lactose, it must be the open conformation that was stimulating lactose transport by $MalFGK_2$. In the mechanistic scheme in Fig. 4, open unliganded MBP would be expected to stabilize the ATP-bound, open-to-periplasm conformation of $MalFGK_2$, perhaps promoting ATP hydrolysis which would then destabilize the tight $MalK_2$ dimer and push the cycle forward.

6. Conclusion: reversibility and a third function for MBP

In this review, I have focused on interactions between MBP and $MalFGK_2$ and attempted to place them in the context of a mechanism for maltose transport. Since there is a requirement for conformational changes in both MBP and $MalFGK_2$, the interactions between them must be dynamic, driving structural changes that promote ATP binding and hydrolysis by the system. For this type of dynamic interaction to occur, MBP must bind to unstable conformations of $MalFGK_2$. I propose that the closed conformation of MBP interacts with a high-energy conformation of $MalFGK_2$ that, in terms of structure, is between the open-to-cytoplasm and open-to-periplasm forms. The high energy of this conformation is most likely due to unfavourable interactions in MalF and MalG that can be overcome, to some degree, by mutation: mutations that lower the energy of this intermediate

conformation decrease the dependence of the system on MBP, producing MBPi–MalFGK$_2$ systems. To allow the transfer of maltose from MBP to MalFGK$_2$, it is logical to posit that the open conformation of MBP interacts with the open-to-periplasm conformation of MalFGK$_2$, and there is evidence to support this idea [51,52]. Furthermore, with ATP occluded in the MalK subunits and poised for hydrolysis, the open-to-periplasm conformation also represents an unstable form of MalFGK$_2$. In conclusion, both closed liganded MBP, and open unliganded MBP interact with unstable conformations of MalFGK$_2$, and so the interactions drive conformational changes in the system that lead to transport of maltose.

An important point with the proposed mechanism and the role for MBP–MalFGK$_2$ interactions is that ATP hydrolysis by the system is only indirectly coupled to maltose transport. Why has this type system evolved instead of an alternative in which ATP hydrolysis is coupled directly to solute transport? One possibility is that the requirement for the closed conformation of MBP on the periplasmic side makes the transport cycle unidirectional. That is, each of the steps in any transport mechanism should, in principle, be reversible, and so the whole cycle should also be reversible. In fact reversibility in an ABC export system has been demonstrated for the multidrug transporter LmrA [68]. For nutrient import systems in bacteria, which must survive in wide range of environments, reversibility would place the organism at a selective disadvantage under nutrient-deprived conditions. The requirement for a closed conformation of MBP to stabilize a high-energy intermediate of MalFGK$_2$ means that, although the system could in principle operate in reverse, in practice this would never occur because it would require a closed, unliganded form of MBP to be present on the periplasmic side. MBP, and PBPs in nutrient import systems in general, may help the system function as a "molecular ratchet", consistent with the ability of these systems to establish very high solute concentration gradients.

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